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Effect of Variation in the Common "a" Determinant on the Antigenicity of Hepatitis B Surface Antigen

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Antibody to the common "a" determinant of hepatitis B surface antigen (HBsAg) protects against infection with hepatitis B virus. A number of variant surface antigens with amino acid substitutions within the "a" determinant have been described in patients around the world. Both wild type and variant HBsAgs were expressed in the yeast *Pichia pastoris* and the antigens were semi-purified and quantitated. The effect on antigenicity of these changes was investigated in a quantitative fashion using four monoclonal antibodies known to bind to different epitopes within the common "a" determinant. The results suggest that amino acid substitution of T131I, K141E and G145R and insertion of 3 amino acids between residues 123 and 124 markedly affect the antigenic structure of HBsAg. These substitutions and insertions in the viral envelope may lead to evasion of the virus neutralizing antibody response and also to reduce efficiency of detection by immunoassays used for diagnosis and blood-bank screening. ***J. Med. Virol. 60:113–121, 2000.***

KEY WORDS: variant hepatitis B virus; immunoassays; diagnosis

INTRODUCTION

Hepatitis B surface antigen (HBsAg) induces an immune response which protects from infection with hepatitis B virus (HBV), a response which is elicited in 95-99% of young HBsAg vaccine recipients. This figure is lower in immunocompromised recipients and in adults of over 40 years of age [Lemon and Thomas, 1997]. The appearance of antibody to HBsAg (anti-HBs) after an infection with HBV indicates successful clearance of the virus and long-term protection. The region of the HBsAg which induces this protective response is common to all subtypes of the virus, it was defined serologically and is designated the "a" determinant. This is considered to lie within the hydrophilic region of the protein between amino acids 110 and 160. Within this region the area around amino acids 124 and 149, which has 5 cysteine residues essential to the immunogenicity of HBsAg, seems to be the most important [Mangold and Streek, 1993]. The "a" determinant contains at least 4 distinct epitopes and 4 overlapping epitopes [Waters et al., 1991] and antibodies to this region confer protection against all subtypes of HBV. There are 9 serological subtypes of HBsAg [Courouce et al., 1976] and nucleotide sequences from the HBsAg coding region have been used to classify HBV into 6 genotypes [Okamoto et al., 1988; Norder et al., 1992]. Molecular studies have shown that there is low variability of the HBsAg gene within the same geographical area and genotype [Norder et al., 1993].

Variations in the primary structure of the "a" determinant have been shown to change markedly the predominantly conformation dependant antigenic structure of HBsAg. A variant virus with an amino acid substitution at residue 145 from glycine to arginine (G145R) was the first described in a child born to a carrier mother. The child was both actively and passively vaccinated at birth and had developed an adequate anti-HBs response [Carman et al., 1990]. The binding of both monoclonal and polyclonal anti-HBs antibodies was affected by this single change and antibody raised to the wild-type HBsAg bound less well to G145R HBsAg [Waters et al., 1992]. This variant has now been described in vaccinated children born to carrier mothers around the world [Oon et al., 1995; Nainan et al., 1996; Lee et al., 1997; Okamoto et al., 1992; Fujii et al., 1992] and has been detected in some of the mothers of these children [Nainan et al., 1996; Lee et al., 1997]. This particular variant has also been found in liver transplant recipients treated with anti-HBs to prevent re-infection of their graft [McMahon et al., 1991; Carman et al., 1996].

In vaccination trials in the United States of 1,092

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TABLE I. Sequences of oligonucleotide primers used for PCR and sequencing*

Primer name	DNA sequence	Nucleotide binding site
Pol.1	5′GCA AAG TTC CCC AAC TTC	895–912
Pre-S2	5′TAT GAG CTC ATG CAG TGG AAT TCC ACT	3171–3188
S1-EcoRI	5′GGA CAG AAT TCA TGG AGA ACA TCA CAT CAG	155–175
S4-EcoRI	5′CTT CTT AAG GGG TTT AAA TGT ATA CCC AGA GAC	818–850
S3	5′CAA GGT ATG TTG CCC GTT TG	457–476

*The numbering of nucleotides is from the unique *Eco R1* site of HBV.

infants born to HBeAg positive mothers 94 became HBsAg positive, and of these infants 22 (23%) had amino acid changes in the region from amino acid 124 to 147. Of these, 8 children had the G145R change [Nainan et al., 1996] and the majority had changes in the 143–145 region.

Other variants of HBsAg have been described. These include a lysine to glutamic acid substitution at residue 141(L141E) in two vaccinated children in the Gambia [Karthigesu et al., 1994]. Of 6vaccinated children in Taiwan who developed variant HBsAg, 4 had the G145R change, 1 had an amino acid change from asparagine to alanine at residue 144 (D144A) and 1 had a methionine to leucine substitution at residue 133 (M133L) [Lee et al., 1997]. The insertion of additional amino acids immediately prior to amino acids 122 and 124 has also been described [Yamamoto et al., 1994; Hou et al., 1995; Carman et al.,1995].

The effect on antigenicity of some changes in the amino acid sequence of the "a" determinant, has been studied experimentally. Mutation of proline 142 reduced antigenicity [Ashton-Rickard and Murray, 1989a] and the cysteine molecules found in the "a" determinant were shown to be essential for full antigenicity [Bruce and Murray, 1995]. Recently the effect of variation at amino acids 126 and 129 on the antigenicity of the HBsAg has been investigated [Chiou et al., 1997], but the remaining variants have not been investigated in this way.

The variants of HBsAg described so far, have in most cases been identified following amplification by nested polymerase chain reaction (PCR). In most of these cases, therefore, the virus load is very low. As a consequence it is not clear whether failure to detect variant HBsAg is truly due to amino-acid substitution or HBsAg being present at concentrations below the minimum detection level of diagnostic kits. It is essential to establish the antigenicity of variant HBsAgs, using recombinant proteins expressed at sufficiently large amounts. In this respect, we undertook to evaluate the effect on antigenicity of substitutions at amino acid positions 131, 141, 142, 145, and 146, which have been found to occur in HBsAg in the presence of an anti-HBs response, and the effect of 2 and 3 amino acid insertions at positions 122 and 123, to determine whether these changes allowed the virus to evade binding of the antibodies which constitute the protective immune response. The entire coding regions for the major (small) HBsAg with wild-type and variant DNA sequences were amplified and cloned in a yeast expression vector. Recombinant proteins were investigated for their antigenic specificities using 4 previously described anti-HBs monoclonal antibodies designated RFHBs 1, 4, 7, and 18 which bind to different epitopes within the "a" determinant [Waters et al., 1992].

MATERIALS AND METHODS

Sources of HBV Variants

Sera from patients with atypical serological findings were investigated for the presence of HBV-DNA by PCR amplification of HBsAg encoding sequences. Carriers of HBV variants were identified among: (a) vaccinated children with low-titre or non-existent anti-HBs born to HBV infected mothers (isolates P142S/G145R and G145A); (b) an HBsAg positive liver transplant patient treated with anti-HBs (F134Y/G145R); (c) an anti-HBs positive patient with chronic hepatitis T131I; (d) a patient with chronic hepatitis who had seroconverted (HBsAg to anti-HBs) following interferon treatment (P142S/G145R/N146D); and (e) 2 chronic HBV carriers with a 2-amino acid or 3-amino acid insertion (122-RA and 123-RGA, respectively). In addition, some of the amino acid substitutions occurring in combination in the isolates were individually engineered by site-directed mutagenesis in an *adr* subtype background (K141E, P142S and G145R).

Two wild-type HBsAg sequences encoding different subtypes were also cloned for use as controls in the epitope mapping studies. The HBV DNA used for these clones came from two HBsAg positive carriers (isolates *wt* adr and *wt* adw).

HBV DNA Extraction From Serum, PCR, and Sequencing

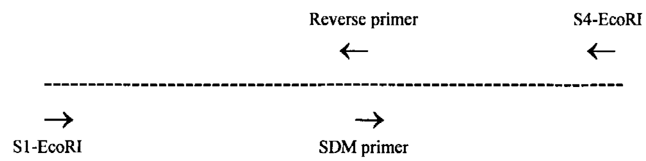
HBV DNA was extracted from 100 µl of serum as described previously [Hou et al., 1995]. After washing twice in 70% ethanol the DNA was resuspended in 10 µl of water. 5 µl of the extracted DNA was amplified by PCR using the oligonucleotide primer pair Pol.1and Pre-S2.. 2 µl of the product was further amplified in a nested PCR using the primers S1-EcoRI and S4-EcoRI. The last two primers contain *Eco RI* recognition sequences to facilitate cloning.

All PCR products were directly sequenced with the Sequenase version 2 kit (Amersham, Little Chalfont, Bucks.) using the outer primers and S3.

The primers used are all listed in Table I.

Cloning and Expression of the Variant Proteins

The HBV DNA amplicons were cloned into the pCRII vector from a TA cloning kit (Invitrogen, Leek, The



SDM Primers.		**Nucleotide binding site**
p.K141E	: 5'TGT TGC TGT ACA **GAA** CCT TCG.	565-585
p. P142S	: 5'TGT TGC TGT ACA AAA **TCT** TCG GAC.	565-588
p.G145R	: 5'TGT TGC TGT ACA AAA CCT TCG GAC **AGA** AAC TGC.	565-597
Reverse primer:	5'TGT ACA GCA ACA AGA.	562-576

Fig. 1. Production of variants by site-directed mutagenesis (SDM). The numbering of nucleotide is from the unique *Eco RI* site of HBV.

Netherlands). The cloned amplicon was released by *Eco RI* digestion, and sub-cloned into the *EcoRI* site of the shuttle vector pHILD2. The DNA sequences of the cloned HBV DNA were confirmed by resequencing using the Sequenase kit, as above.

The recombinant vector was linearised by digestion with *SacI* restriction enzyme and electrocompetent yeast cells of *Pichia pastoris* strain KM71 were transformed using the one step gene insertion method [Scorer et al. 1994]. Both plasmids and the yeast cells were purchased from Invitrogen, as above.

Single colonies of the transformed yeast cells were isolated using the methods recommended by the manufacturers. HBV DNA positive colonies were identified by hybridisation with wild-type ^{32}P-labelled cloned “S” gene DNA. Recombinant yeast cells from colonies which gave a high signal, which may reflect the copy number integrated into the yeast genome, were grown in baffled shaking-flasks, expression was induced with 1% methanol as a carbon source. After this time the yeast cells were pelleted and the cell walls disrupted by vortexing with 400–600 micron glass beads (Sigma Chemical, Poole, UK). The cell debris was pelleted by centrifugation at 10,000g for 10 minutes and the cell extract sonicated six times in bursts of 30 seconds on ice. The presence of HBsAg in the yeast cell extract was confirmed by testing in the AUSRIA II assay (Abbott Diagnostics, Queenborough, UK).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed as described by Ho et al. [1989]. Briefly, the cloned wild type *adr* PCR product was used as the DNA template for generating fragments with a single nucleotide change incorporated in complementary oligonucleotide primers. PCR was used to generate two DNA fragments with complementarity over the primers and with the mutations. These fragments were combined in a subsequent splicing reaction in which the overlapping ends annealed, allowing the 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The resulting fusion product was amplified further by PCR using the outer primers defining the entire amplified region (Fig. 1).

Southern and Northern Blotting

Total genomic DNA was isolated from a 10 ml culture of each of the yeast clones [Ausubel et al., 1990]. 5 μg of DNA was digested with *EcoRI,* separated on a 1% agarose gel and transferred to a Hybond Nylon membrane (Amersham International, Little Chalfont, UK). Known quantities of a standard DNA preparation of the wild type S gene (10, 5, 2, 1, 0.1ng) were also included. The DNA was probed with ^{32}P-labelled “S” gene DNA. Northern blotting was performed by isolating mRNA from the yeast clones using the method of Schmitt et al. [1990], separating the RNA on a 1% formaldehyde gel, transferring to a membrane and probing, as above.

Semi-Purification and Quantification of HBsAg

Semi-purification of HBsAg from the yeast extracts was performed by ammonium sulphate precipitation, a modification of the method of Ashton-Rickard and Murray [1989b]. Briefly, an initial precipitation with 20% saturated ammonium sulphate (110 g/L) was followed by a second precipitation with 45% saturated ammonium sulphate (a further 137 g/L). The second precipitate was resuspended in 1 ml PBS.

Semi-purified HBsAg from the *wt*-adr clone was run on a 12.5% SDS-PAGE gel, transferred to nitrocellulose membrane and HBsAg bands were detected by Western blotting with the murine monoclonal antibody, RFHBs 7 [Waters et al., 1991]. The antigen content of this preparation was measured against the standard HBsAg using the AUSRIA II kit. Known volumes of all of the wild type and variant HBsAg preparations were separated by SDS-PAGE and stained with silver stain. The density of the unglycosylated HBsAg band was measured and compared to that of the “*wt*-adr” clone using a computer program that enabled the amount of

the variant HBsAg extracted to be calculated (Quanti-Scan, Biosoft, Cambridge, UK).

Characterization of Recombinant HBsAg

HBsAg preparations expressed by clones *wt*-adr and F134Y/G145R were centrifuged through a 20%–50% sucrose gradient in a buffer containing 0.14M NaCl, 1mM EDTA and 50 mM sodium phosphate pH 7.4, for 20 hours at 5°C at 29,000 rpm in a Sorvall AH629 rotor. All fractions were tested in the AUSRIA II assay. The density of the fractions was also measured using a refractometer. The fraction with HBsAg reactivity was dialyzed against water and then concentrated on a bed of PEG 6000. This material was used to prepare grids which were examined by immuno-electron microscopy.

Epitope Mapping of the Wild Type and the Variant HBsAgs

Polystyrene beads were coated with individual murine monoclonal antibodies RFHBs 1, 4, 7, or 18 as described previously [Goodall et al., 1981]. The monoclonal antibody coated beads and polyclonal guinea pig anti-HBs coated beads from AUSRIA II kit were incubated overnight with 10, 5, 2, 1, and 0.5 ng/ml of the semi-purified wild-type and variant HBsAgs. The beads were washed and the amount of HBsAg bound was detected using the radio labeled human anti-HBs from the AUSRIA II kit.

RESULTS

Sequencing, Southern and Northern Blotting of the Yeast Clones

The entire HBsAg coding region was sequenced from all isolates. The sequences of the "a" determinant of all of the clones, both wild-type and variant are listed in Table II. The genotype of each isolate is listed in Table III.

Southern blot analysis of restricted yeast genomic DNA released a band of the predicted size of 680 bp. In addition, Northern blot analysis confirmed production of S-gene RNA transcripts of about 1000 bp by all recombinant yeast constructs.

Semi-Purification and Quantification of HBsAg

The yield of HBsAg obtained by ammonium sulphate precipitation of the extract from 1.5×10^{10} yeast cells expressing *wt*-adr was estimated to be 7μg. The two major bands of HBsAg of molecular weights 24 kD and 28kD were identified by Western blotting with RFHBs 7 (Fig. 2). These sizes accord well with the size of the glycosylated and unglycosylated plasma derived HBsAg. HBsAg prepared by HPLC using the same yeast construct and kindly purified by Boehringer Mannheim (Mannheim, Germany) had the same size bands. RFHBs 7 did not bind to the semi-purified extract of the parental yeast strain. The 24kD band of each of the semi-purified extracts of the yeast preparation expressing the variant HBsAgs, were identified on the silver stained SDS-PAGE gels by comparison to the *wt*-adr preparation (Fig. 3). There was no band at that position in the extract from the parental yeast. The total amounts of variant HBsAg, purified from the same number of yeast cells, are listed in Table III.

Characterization of Recombinant HBsAg

The peak fraction from the sucrose gradient of both *wt*-adr and F134Y/G145R had densities of 1.16 g/ml. This density is similar to the density of serum derived HBsAg [Gerin et al., 1969]. Electron microscopy of the *wt*-adr HBsAg identified particles 22 nm in diameter (not shown).

Comparative Antigenicity of the Wild-Type and Variant HBsAgs

Variant HBsAgs T131I, P142S/G145R and P142S/G145R/N146D were undetectable using the AUSRIA II kit. Variants T131I and P142S/G145R were expressed at a concentration of 250ng/ml and 310ng/ml respectively and were undetectable by the AUSRIA II assay even at this concentration. P142S/G145R/N146D was expressed at a concentration of 14ng/ml and at this concentration was undetectable. The variant 123-RGA was also expressed at a low concentration (7 ng/ml) and was not detectable by AUSRIA II. All of these variants were also not detectable using the monoclonal solid phases; this may have been due to lack of binding by the anti-HBs tracer.

The variant 122-RA was detectable using the monoclonal antibodies RFHBs 1, 4, and 18. This protein was also produced in a very low concentration (4.6 ng/ml) but there was no decrease in reactivity with RFHBs 1 and 18 and an increase in reactivity with RFHBs 4. This variant was also detectable at this concentration using the AUSRIA II assay.

The remaining variants were also tested using the monoclonal antibody coated solid phases and the binding curves are illustrated in Figure 4. Variant K141E was detectable by the AUSRIA II assay but with over a thousand fold loss of sensitivity. Using the anti-"a" determinant monoclonal antibodies RFHBs 1 and RFHBs 7, this variant was also just detectable at the same concentration, giving a positive to negative ratio of 2 at a concentration of 400 ng/ml.

The variant G145R was detectable to a concentration of 2 ng/ml using the RFHBs 4 solid phase, but was not bound by RFHBs 1, 7, and 18. However the F134Y/G145R variant was detectable by RFHBs 1 to a concentration of 5 ng/ml and to a concentration of 1ng/ml using the RFHBs 4 solid phase. The antibodies RFHBs 7 and 18 did not bind to this antigen even at a concentration of 10 ng/ml.

The variant P142S/G145R was also not bound by the monoclonal antibodies or the AUSRIA II assay even at a concentration of 310 ng/ml but the protein containing a change of proline for serine at amino acid 142 alone was detectable using all of the monoclonal antibodies; there was some loss of reactivity with RFHBs 18 but an increase in binding by RFHBs 4. At residue 145 a different amino acid substitution G145A had a less marked effect on binding by the monoclonal antibodies:

TABLE II. Predicted Amino Acid Sequences of the “a” Determinant of the Recombinant HBV Variants

	120										130										140										150										160
Consensus (adr)	P	C	K	T	C	T	I	P	A	Q	G	T	S	M	F	P	S	C	C	C	T	K	P	S	D	G	N	C	T	C	I	P	I	P	S	S	W	A	F	A	R
***wt*-adr**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K141E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P142S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G145R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F134Y/G145R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Consensus-ayw	P	C	R	T	C	M	T	T	A	Q	G	T	S	M	Y	P	S	C	C	C	T	K	P	S	D	G	N	C	T	C	I	P	I	P	S	S	W	A	F	G	K
T131I	-	-	-	-	-	T	-	P	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Consensus-adw	P	C	K	T	C	T	T	P	A	Q	G	N	S	K	F	P	S	C	C	C	T	K	P	T	D	G	N	C	T	C	I	P	I	P	S	S	W	A	F	A	K
***wt*-adw**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P142S/G145R	-	-	-	-	-	-	-	-	-	-	-	T	-	M	-	-	-	-	-	-	-	-	S	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G145A	-	-	-	-	-	-	-	-	-	-	-	T	-	M	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
146*	-	-	-	-	-	-	-	-	-	-	-	T	-	M	-	-	-	-	-	-	-		S	-	-	R	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-
122-RA	-	-	-	-	-	-	-	-	-	-	-	T	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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123-RGA	-	-	-	-	-	-	-	-	-	-	-	T	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
					R	G	A																																		

*146 recombinant HBsAg P142S/G145R/N146D.

TABLE III. Subtype, Genotype and Expression of Recombinant HBsAg Variants

Variant	Subtype	Genotype	HBsAg expressed by 1.5×10^{10} cells (ng)
WT-adr	adr	C	7000
K141E	adr	C	400
P142S	adr	C	830
G145R	adr	C	240
F134Y/G145R	adr	C	230
T131I	ayw4	D	250
WT-adw	adw2	B	320
P142S/G145R	adw2	B	310
G145A	adw2	B	33
P142S/G145R/N146D	adw2	B	14
122-RA	adw2	B	4.6
123-RGA	adw2	B	7

it was detectable using RFHBs 1 and 4 but not by RFHBs 7 and 18 on the solid phase.

DISCUSSION

The tertiary structure of HBsAg, especially of the immunodominant "a" determinant, is crucial for its recognition by antibody. This is important since antibodies to the "a" determinant protect against infection with HBV and variants which are no longer recognised by these antibodies will evade this protective immune response. Antibodies to HBsAg are also widely used in assays for diagnosis of HBV infection, and for screening of blood and its products for HBV infection. Therefore, variants with a sufficiently altered structure which are poorly recognized or unrecognized by these assays, pose a risk. It is also important to establish beyond any doubt that failure to detect variant HBsAgs is due to the antigenic changes described and not due to very small amounts of HBsAg present, an eventuality which cannot be excluded, since most of these changes may also affect the overlapping polymerase ORF and therefore the replication capacity of the virus.

The expressed *wt*-adr and F134Y/G145R HBsAgs were confirmed as having a particulate structure with the same density and size as material derived from the plasma of infected individuals. The recognition of our recombinant *wt*-adr and *wt*-adw by the anti-"a" determinant monoclonal antibodies also confirmed the presence of the correct conformational epitopes as in plasma derived HBsAg.

The antigenic structure of the "a" determinant has been mimicked in part by two cyclical peptide analogues of amino acids 124–137 and 139–147 [Howard et al., 1984]. These cyclical peptides were a convenient way to investigate the epitopes recognized by monoclonal antibodies. However, the disulphide bonds between cysteine residues which are formed within or between HBsAg molecules (intra- and intermolecular bonds) remain unknown. The antibodies used in this study were selected for their binding to these peptides. RFHBs 1 and 4 bind to the peptide 124-137 and RFHBs 7 binds to 139-147 although with reduced affinity in compari-

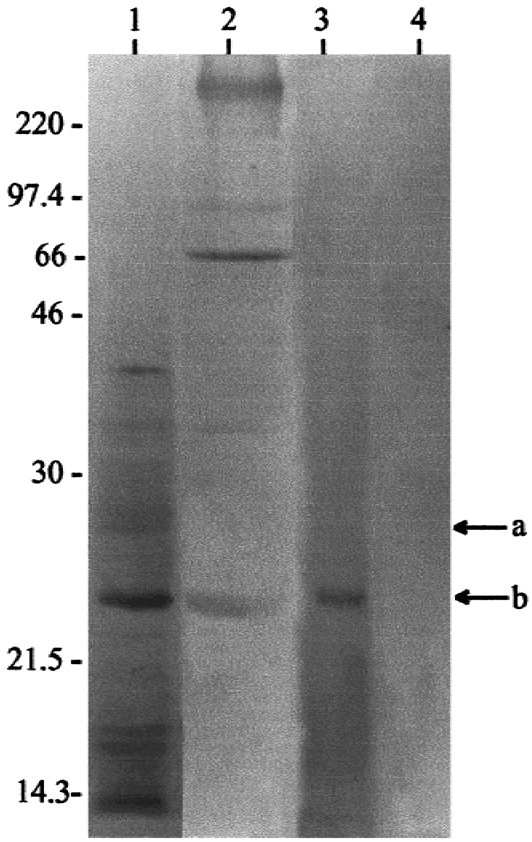

Fig. 2. Western blot recombinant *wt*-adr HBsAg preparations with the monoclonal antibody RFHBs 7. **1.** *wt*-adr, ammonium sulphate precipitated; **2.** *wt*-adr, HPLC-purified; **3.** HBsAg, positive control; **4.** KM-71 (yeast negative control).

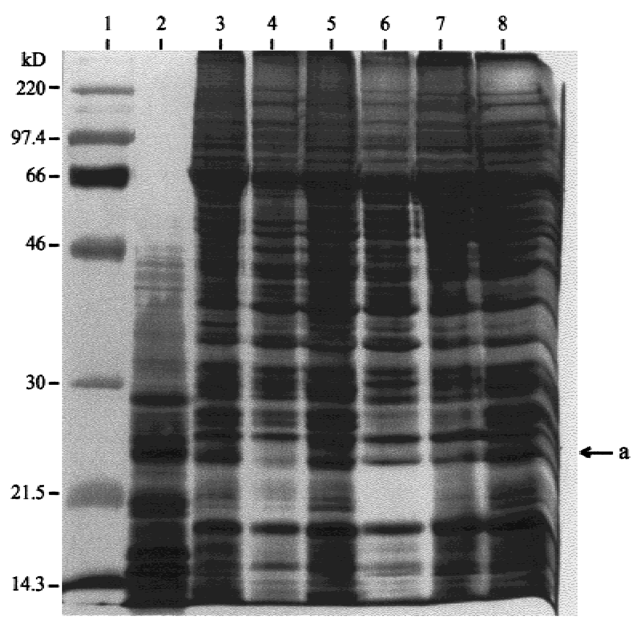

Fig. 3. Silver stained SDS-PAGE of extracts from recombinant yeast clones partially purified by ammonium sulphate precipitation. **1.** Molecular weight markers; **2.** *wt*-adr; **3.** *wt*-adw; **4.** 122-RA; **5.** F134Y/G145R; **6.** G145A; **7.** P142S/G145R/N146D; **8.** T131I

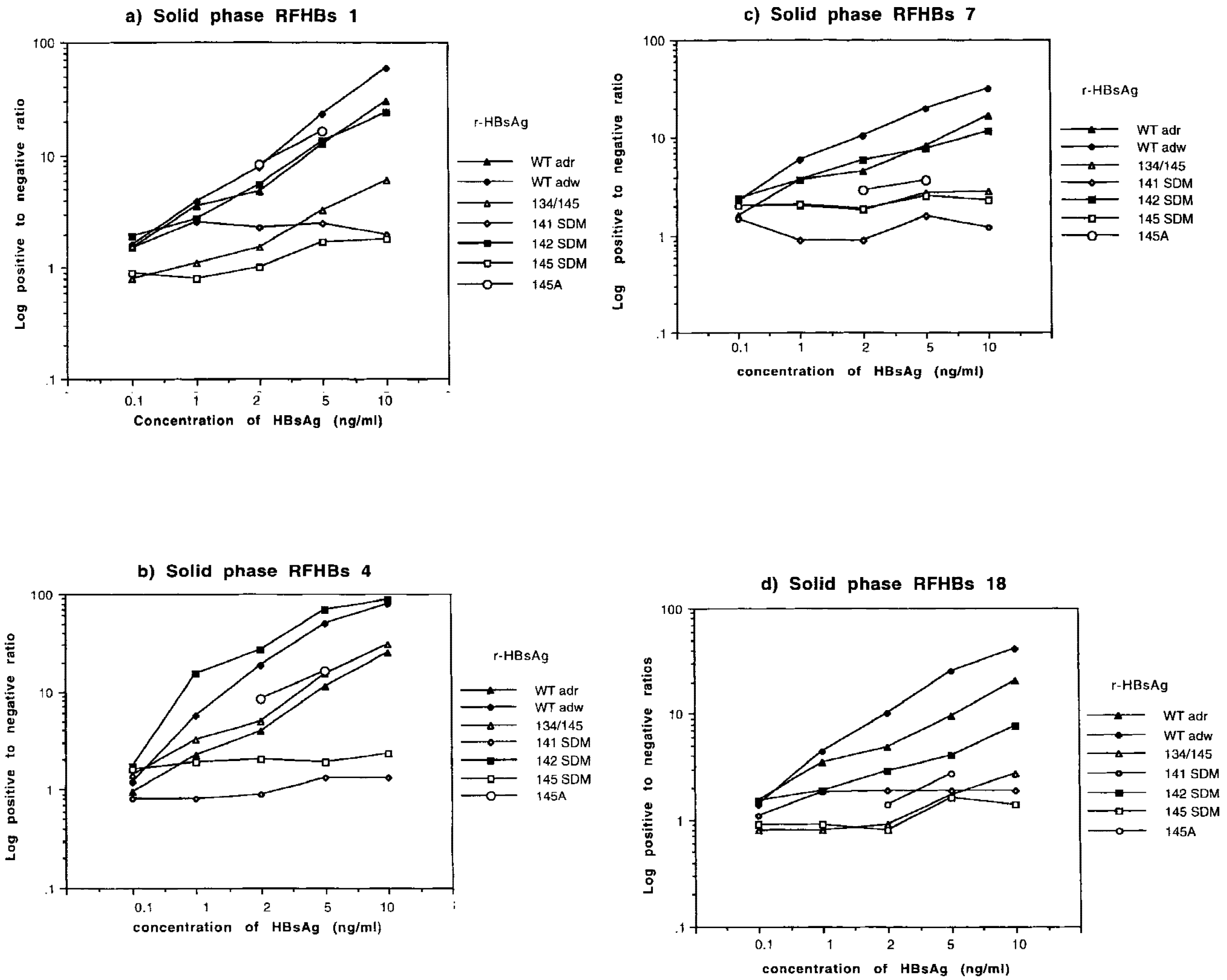

Fig. 4. Epitope mapping of recombinant HBsAg variants by the anti-“a” determinant monoclonal antibodies RFHBs 1, 4, 7, and 18. The monoclonal antibodies were coated onto the solid phase and incubated with dilutions of the ammonium sulphate precipitated HBsAgs, the amount bound was detected with radio labeled anti-HBs. The results are expressed as the log of the positive to negative ratio.

son to their binding to the solubilised native molecule [Waters et al., 1991]. RFHBs 18 partially cross-competes with RFHBs 7. All of the antibodies recognise different epitopes within the “a” determinant in that they do not completely inhibit the binding of each other. In addition, RFHBs 1 has been shown to neutralise HBV infectivity in chimpanzee challenge studies using both ad and ay standard HBV inocula [Iwarson et al., 1985] and therefore is also active in the ayw background.

The G145R substitution, which was the first of the HBsAg variants to be described [Carman et al., 1990], has been shown to affect the antigenic structure of the whole of the “a” determinant [Waters et al., 1992]. This was confirmed in this study: there was loss of binding of RFHBs 1, 7 and 18 and reduced binding by RFHBs 4 to the recombinant protein expressed by the G145R construct in comparison to the binding to *wt*-adw or *wt*-adr. However, an additional amino acid change, F134Y, in part restored recognition by antibodies specific for HBsAg. Restoration of the binding to RFHBs 1 although not to equivalence with the wild-type HBsAg, suggests that this second mutation restores the conformation of the part of the molecule represented by this cyclical peptide 124-137, and partly compensates for the structural changes caused by the G145R change.

Loss of the proline at amino acid position 142 in conjunction with the G145R change resulted in total loss of reactivity with both monoclonal and polyclonal anti-HBs antibodies. The effect of the P142S change alone was not so marked. There was some loss in binding by two of the monoclonal antibodies, RFHBs 1 and RFHBs 18, and a marked increase of binding by RFHBs 4 suggesting that a change at amino acid 142 does affect the structure of the “a” determinant. In particular the epitope recognised by the monoclonal antibody RFHBs 4 either becomes more exposed or the epitope is changed in a way which increases the affinity of the antibody for the antigen. This antibody binds to the cyclical peptide analogue 124–137 as does RFHBs 1 but only partially

competes with this antibody when binding to the native molecule. The variant protein which had the same substitutions at amino acid positions 142 and 145 plus an additional change of N146D could also not be detected at the same concentrations at which the *wt* antigens were detectable.

The amino acid substitution to arginine at position 145 would appear to have the greatest effect on the antigenicity of HBsAg since the results also indicate that a substitution to alanine reduces the binding of the monoclonal antibodies but to a lesser extent than the change to arginine. The binding of RFHBs 4 was not affected, but reduced binding by RFHBs 1, 7 and 18 suggests that this amino acid change also alters the conformation of the whole of the "a" determinant.

The amino acid residues at positions 131 and 141 were also shown to be important for the detection of HBsAg by immunoassay. Substitutions at these positions (T131I and K141E), even at high concentrations of antigen, resulted in loss of binding by all of the monoclonal and polyclonal antibodies. Similarly, insertion of 3 amino acids immediately before the cysteine residue at position 124, adversely affected the binding and therefore the detection of this variant protein by antibody, whereas a 2 amino acid insertion between 122 and 123 had a lesser effect on recognition by antibody. The cysteine residues 121 and 124 are highly conserved among hepatitis viruses and replacement of these residues separately resulted in loss of recognition by antibodies [Antoni et al., 1994]. This group suggested that the disulphide bond in which the cysteine residue C124 is involved is more important for the conformation and antigenicity of HBsAg than that involving C121 since binding by antibody was more seriously disrupted when this cysteine was replaced. Also on the basis of a monoclonal antibody binding it has been suggested that C121 and C124 form a disulphide bond [Chen et al., 1996]. Additional amino acids inserted between C121 and C124 may disrupt the antigenic structure of HBsAg but the greater loss of antigenicity caused by the 3 amino acid insertion immediately adjacent to C124 may also be due to its position.

It is apparent that some variants can be detected by using the right combination of monoclonal and polyclonal antibodies with however, in many cases, reduced sensitivity. It follows that the presence of such variant antigen in serum below a critical concentration will lead to failure in detection. Such an outcome would have repercussions in blood bank testing, particularly if these variants were as replication competent as the wild-type, and became widespread. Since the nucleotide substitutions that affect the "a" determinant are also part of the overlapping polymerase open reading frame, it remains possible that they may also influence polymerase function. These variants may also evade the vaccine induced immune response to small HBsAg vaccines. However, two existing recombinant vaccines were able to protect against infection with the variant G145R in chimpanzee challenge experiments [Ogata et al., 1993], although in mice the titre of antibody which bound to the variant after immunization with the wild-type antigen was reduced [Waters et al., 1992]. High titre antibody raised by vaccination may protect against infection by variant viruses and protection may be enhanced by inclusion of pre-S or core antigen into recombinant vaccines. Although the G145R variant has been shown to replicate in the chimpanzee [Ogata et al., 1997], the efficiency of replication of the other variants has not been investigated.

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